

Induction of Lung and Exocrine Pancreas Tumors in F344 Rats by Tobacco-specific and *Areca*-derived *N*-Nitrosamines^{1,2}

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ABSTRACT

The tobacco-specific *N*-nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), as well as the *Areca*-derived *N*-nitrosoguvacoline (NG) were assayed for carcinogenicity in male F344 rats by lifetime administration in the drinking water. Groups of 30 to 80 rats were treated with 0.5 ppm, 1.0 ppm, or 5.0 ppm of NNK; 5.0 ppm of NNAL, 20 ppm of NG, a mixture of 20 ppm of NG and 1 ppm of NNK, and water only in the control group. The approximate total doses of the nitrosamines (mmol/kg of body weight) in these groups were: NNK, 0.073, 0.17, and 0.68; NNAL, 0.69; NG, 4.1; NG and NNK, 4.1 and 0.17. As in previous assays in which NNK was tested by s.c. injection, the lung was its principle target organ. Lung tumor incidences in the 0.5-, 1.0-, and 5.0-ppm groups were nine of 80, 20 of 80, and 27 of 30 compared to six of 80 in the control rats. This trend was significant, $P < 0.005$. Significant incidences of nasal cavity and liver tumors were observed only in the rats treated with 5.0 ppm of NNK. In contrast to the results of the s.c. bioassays of NNK, tumors of the exocrine pancreas were observed in five of 80 and nine of 80 rats treated with 0.5 and 1.0 ppm. This trend was significant, $P < 0.025$. This is the first example of pancreatic tumor induction by a constituent of tobacco smoke. It is also the first finding of duct-like carcinomas in the rat pancreas, including one tumor containing epidermoid, keratin-generating tissue. NNAL, the major metabolite of NNK, induced lung tumors in 26 of 30 rats and pancreatic tumors in eight of 30 rats. It appears to be the proximate pancreatic carcinogen of NNK. NG induced pancreatic tumors in four of 30 rats, $P < 0.05$. This finding requires confirmation. The mixture of NG and NNK induced lung tumors in eleven of 30 rats. There were no apparent synergistic interactions of NG and NNK. The observation of benign and malignant tumors of the lung and pancreas of rats treated with the tobacco-specific nitrosamines NNK and NNAL is discussed in respect to the causal association between cigarette smoking and cancer of the lung and pancreas.

INTRODUCTION

Epidemiologically, cigarette smoking is causally linked with cancer of the lung, larynx, oral cavity, esophagus, pancreas, renal pelvis, and urinary bladder and is also associated with cancer of the nasal cavity and cervix. Smoking of cigars and pipes is causally related to cancer of the respiratory tract, oral cavity, and esophagus, although, in the case of lung cancer, not to the same extent as cigarette smoking (1-3). Chewing of tobacco, and especially the oral use of snuff, is associated with cancer of the oral cavity and, possibly, with cancer of the nasal cavity, pancreas, kidney, and bladder (4-8). The habit of chewing tobacco-containing betel quid is known to lead to cancer of the mouth and of the esophagus (4).

The TSNA⁴ (Fig. 1) are the most abundant, strong carcino-

gens in chewing tobacco, snuff, tobacco-containing betel quid, and tobacco smoke (4, 6, 9, 10). They are formed by *N*-nitrosation of nicotine (1 to 2% of the tobacco) during processing and storage. In cigarette smoke, 26 to 37% of NNK and 40 to 46% of NNN originate from tobacco by direct transfer; the remainder is pyrosynthesized during smoking (11, 12). The *N*-nitrosamines generated as a result of the *N*-nitrosation of arecoline, the major alkaloid in betel quid, are NG, NGC, MNPN, and MNPA (Fig. 2; Ref. 13).

The levels of NNN and NNK in chewing tobacco (1 to 8.5 ppm), snuff (3 to 100 ppm), and cigarette smoke (0.2 to 4 μ g/cigarette) are generally at least two orders of magnitude higher than the concentrations of carcinogenic *N*-nitrosamines in other consumer products or respiratory environments (10, 14, 15). Mixtures of betel quid and tobacco also contain NNN (0.025 to 0.1 ppm), NNK, and NG (up to 0.014 ppm) (16). NNN and NNK induce benign and malignant tumors in the nasal cavity, oral cavity, esophagus, lung, and/or liver of mice, rats, and hamsters (10, 17), while NNAL causes lung tumors in mice (17). NNK is considered to be more carcinogenic in F344 rats than *N*-nitrosodimethylamine (18, 19). Lijinsky and Taylor reported that NG, the major nitrosation product of arecoline, was not carcinogenic when given to rats in drinking water (20).

NNK, the most carcinogenic compound in the TSNA group, had not been previously tested by administration in the drinking water. Its presence in the saliva of tobacco chewers, tobacco smokers, and chewers of tobacco-containing betel quid (16, 21-23) warranted testing by p.o. application. NNAL, the major metabolite of NNK (24-26), had not been previously tested for carcinogenicity in rats. In this lifetime bioassay, male F344 rats were given 0.5, 1.0, or 5.0 ppm of NNK or 5.0 ppm of NNAL in the drinking water. We also assayed NG (20 ppm) and a mixture of NNK (1.0 ppm) and NG (20 ppm) in a ratio comparable to that found in the saliva of chewers of tobacco-containing betel quid (13, 22).

MATERIALS AND METHODS

Chemicals. NNK, NNAL, and NG were synthesized according to earlier published methods. They were greater than 99% pure according to gas chromatography and high-performance liquid chromatography analyses (26-28). The solutions of the *N*-nitrosamines were newly prepared every 2 wk and were stored in amber bottles in a cold room prior to administration.

Bioassays for Carcinogenicity. Male F344 rats, 6 wk old, were obtained from Charles River Breeding Laboratories, Kingston, NY. When the rats were 8 wk old, the bioassay was started. The rats were housed in groups of 3 in solid-bottomed polycarbonate cages with hardwood bedding under standard conditions [$20 \pm 2^\circ\text{C}$ (SD); $50 \pm 10\%$ relative humidity; 12-h light and dark cycle]. NIH-07 diet and tap water with or without *N*-nitrosamines were given *ad libitum*. The 500-ml amber bottles were filled with the drinking water preparations every seventh day, and the fluid consumption was recorded.

The bioassay consisted of the following groups: I, 0.5 ppm of NNK, 80 rats; II, 1.0 ppm of NNK, 80 rats; III, 5.0 ppm of NNK, 30 rats; IV, 5.0 ppm of NNAL, 30 rats; V, 20 ppm of NG, 30 rats; VI, 20 ppm of NG plus 1.0 ppm of NNK, 30 rats; and VII, negative control group (water only), 80 rats.

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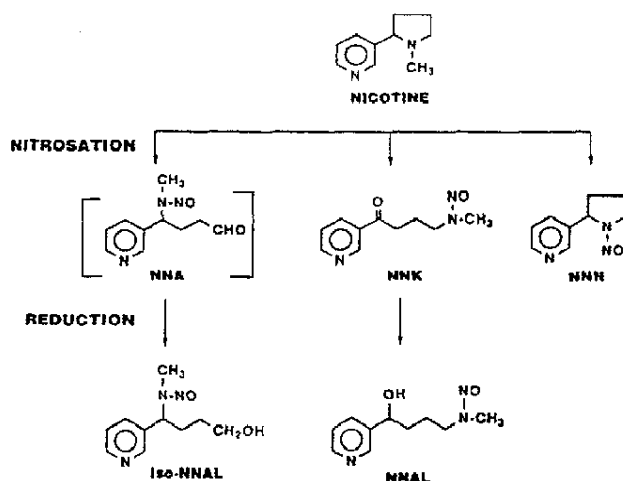
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⁴ The abbreviations used are: TSNA, tobacco-specific *N*-nitrosamines; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; MNPA, 3-(methylnitrosamino)propionaldehyde; MNPN, 3-(methylnitrosamino)propionitrile; NG, nitrosoguvacoline; NGC, nitrosoguvacine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosornicotine.

Fig. 1. Formation of tobacco-specific *N*-nitrosamines.

The rats were observed until moribund or until scheduled termination of the experiment and were killed by CO₂ inhalation. Complete autopsies were performed. Histology slides were prepared for all gross lesions and for lung, liver, spleen, kidney, adrenals, pancreas, esophagus, larynx, and trachea. To search for nasal cavity tumors, the head was decalcified, after removing the skin, mandibula, and muscles. The decalcified head was then cut in 2 parts through a frontal retroorbital section. The anterior part containing the nasal cavity was further divided in 3 fragments by 2 equidistant, frontal cuts. All 3 pieces obtained were embedded in paraffin and processed for microscopic examination.

Statistical evaluations were done with the 2-sample *t* test and χ^2 test. To evaluate the trends for the yields of specific tumors between experimental groups at different dose levels, we used the Bartholomew test (29) and Tukey test for multiple comparisons (30).

RESULTS

Table 1 summarizes data on estimated total doses of *N*-nitrosamines, length of survival, time until termination of the assay, water consumption, and body weights of the rats. Survival of the rats receiving the high dose of NNK (Group III) was significantly shorter than that of the rats receiving water only ($P < 0.01$) and of the rats receiving the lower doses of NNK ($P < 0.05$). The rats in Group III (5.0 ppm of NNK) consumed significantly less water than the rats in Group VII (water only; $P < 0.01$). There were no significant differences in the body weights of the animals in experimental groups and the control group.

Table 2 summarizes data on tumor incidence. A new and important finding was the occurrence of adenomas and adenocarcinomas of acinar and/or ductal origin in the exocrine pancreas of rats receiving 1.0 ppm of NNK (Group II) or 5.0 ppm of NNAL (Group IV). These types of tumors occur only very rarely as spontaneous neoplasms in F344 rats (31).

The diameters of the pancreatic tumors varied from 0.3 to 1.5 cm. Adenomas were smaller than carcinomas. They could be seen with the unaided eye on microscopy slides, as round or ovoidal nodules, contrasting sharply with the polygonal shape of the pancreatic lobules. Histologically, the adenomas were composed of pancreatic acini larger than normal. The absence of Langerhans islets in the tumor mass is a constant diagnostic feature. With hematoxylin-eosin staining, the cell cytoplasm had approximately the same staining characteristics as the normal pancreatic cells, but the nuclei were dysplastic. In

addition, the crowding and "piling up" process, never seen in the normal pancreas, was always present in these acinar tumors. Mitoses were frequently found, and they appeared hyperchromatic and sometimes multipolar.

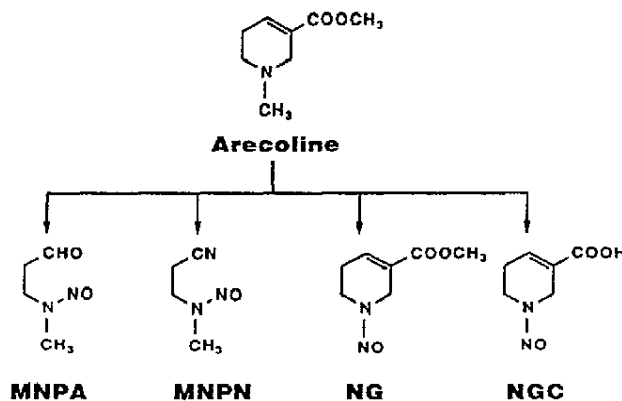
In some larger well-differentiated acinar adenocarcinomas, the acinar pattern was broken by a tendency to form papillary structures. It is noteworthy that the few acinar carcinomas described in the human pancreas had similar patterns (32).

The second type of carcinomas of exocrine origin was formed mostly of duct-like structures of various dimensions and shapes, combined with "solid" areas as well as papillary or trabecular aspects (Figs. 3 to 8). Mucous material was present in and outside the tubular lumina. One of the rats had a pancreatic tumor with intermixed areas of ductal, endocrine, and epidermoid (keratinizing) tissue (Figs. 6 to 8).

Although the number of rats with exocrine tumors of the pancreas in Group I (Table 2) was not significantly different from that in the negative control (Group VII), Bartholomew's test showed a significant positive trend between the control Group VII, Group I (0.5 ppm of NNK), and Group II (1.0 ppm of NNK; $P < 0.025$).

As in earlier bioassays in which we applied it by s.c. injection (19, 20), NNK proved to be a powerful lung carcinogen in rats also when applied p.o. The difference in lung tumor incidence between Group I (0.5 ppm of NNK) and the control group was not statistically significant; yet Bartholomew's test (29) showed a highly significant trend for lung tumors across exposure levels (Groups VII, I, II, and III; $P < 0.005$). NNAL was also a potent lung carcinogen, with activity similar to that of NNK. The morphology of the adenomas and adenocarcinomas in the lung did not differ markedly from that observed after s.c. injections of NNK (18, 19). Epidermoid metaplastic foci occurred in large areas of these tumors and generated squamous carcinomas in greater number than after s.c. injection of NNK.

Surprisingly, the incidence of tumors of the nasal cavity was lower than that observed upon s.c. injection of NNK (18, 19). The incidence of the nasal cavity tumors was significant only in the group treated with the highest dose of NNK (Group III). Liver tumor incidence in this bioassay appears to be similar to that observed in previous assays of NNK (18, 19). Liver tumors were induced in 12 of 30 rats in Group III (total dose, 0.68 mmol/kg) and in 11 of 80 rats receiving 0.17 mmol/kg. In the negative control group, 6 of 80 rats developed liver tumors. Upon s.c. injection, a dose of 0.33 mmol/kg induced liver tumors in 10 of 27 rats (19).

Fig. 2. Formation of *Areca*-derived nitrosamines.

TOBACCO-RELATED TUMORS OF THE LUNG AND PANCREAS IN RATS

Table 1 Uptake of *N*-nitrosamines, survival time, water consumption, and body weights of male F344 rats

	Group I, NNK, 0.5 ppm	Group II, NNK, 1.0 ppm	Group III, NNK, 5.0 ppm	Group IV, NNAL, 5.0 ppm	Group V, NG, 20 ppm	Group VI, NG + NNK, 20 ppm + 1.0 ppm	Group VII, water only
No. of rats	80	80	30	30	30	30	80
Dose, mg/rat	6.9 ± 1.8 ^a	15.6 ± 3.3	63.5 ± 11.6	66.6 ± 14.5	315 ± 68.6	313 ± 60.6 + 15.7 ± 3.0	
Dose, mmol/kg	0.073	0.17	0.68	0.69	4.1	4.1 + 0.17	
Av. survival (wk)	103.3 ± 14.3 ^b	105.1 ± 11.9 ^b	90.1 ± 11.8	93.0 ± 16.2	105.9 ± 17.2	109 ± 13.7 ^c	108.0 ± 11.5 ^c
Termination (wk)	128	120	108	112	128	128	128
Water consumption (liters)	13.8 ± 3.5	15.6 ± 3.3	12.7 ± 2.3 ^d	13.3 ± 2.9	15.8 ± 3.4	15.7 ± 3.0	15.1 ± 3.1
Body wt of rats at wk							
22	389.9 ± 22.2	386.1 ± 18.3	389.3 ± 20.0	390.7 ± 23.2	386.9 ± 21.5	381.1 ± 20.5	385.0 ± 20.4
46	459.0 ± 28.7	454.3 ± 25.2	456.8 ± 25.8	454.6 ± 42.4	460.4 ± 31.1	450.3 ± 26.5	454.6 ± 25.7
65	480.6 ± 32.2	477.6 ± 26.6	477.4 ± 32.6	483.9 ± 30.0	484.1 ± 32.6	475.3 ± 31.2	476.7 ± 29.4
88	494.8 ± 35.9	481.5 ± 35.4	471.8 ± 42.3	487.4 ± 30.9	492.5 ± 38.8	492.7 ± 56.0	483.4 ± 32.9
108	449.0 ± 47.2	423.6 ± 37.0	318.0 ^e	456.2 ± 36.1	434.9 ± 33.7	432.7 ± 42.0	441.3 ± 39.5

^a Mean ± SD.^b Longer survival rates ($P < 0.05$; Groups I and II compared to III).^c $P < 0.01$ (Groups VI and VII compared to III).^d Lower water consumption ($P < 0.01$; Group III compared to Group VII).^e Only 1 rat survived.Table 2 Tumor incidence in male Fischer rats upon administration of *N*-nitrosamines in drinking water

	Group I, NNK, 0.5 ppm	Group II, NNK, 1.0 ppm	Group III, NNK, 5.0 ppm	Group IV, NNAL, 5.0 ppm	Group V, NG, 20 ppm	Group VI, NG + NNK, 20 ppm + 1.0 ppm	Group VII, water only
No. of male F344 rats	80	80	30	30	30	30	80
No. of rats with nasal cavity tumors							
Olfactory tumors	0	1	3	0	1	0	0
Respiratory tumors	1	1	2	0	0	1	0
Total no. of rats with nasal cavity tumors	1	2	5 ^a	0	1	1	0
No. of rats with liver tumors							
Adenomas	2	9	10 ^a	2	0	2	6
Hepatomas	1	2	2	1	0	1	0
Total no. of rats with liver tumors	3	11	12 ^a	3	0	3	6
No. of rats with lung tumors							
Adenomas	5	16 ^a	2	5	2	6	3
Adenocarcinomas	3	4	13 ^a	12 ^a	0	4	2
Adenosquamous carcinoma	0	0	9 ^a	9 ^a	0	1	1
Squamous cell carcinoma	1	0	3 ^a	0	0	0	0
Total no. of rats with lung tumors	9	20 ^a	27 ^a	26 ^a	2	11 ^a	6
No. of rats with exocrine pancreas tumors							
Acinar adenoma	5	8 ^b	1	3	4 ^b	2	1
Acinar adenocarcinoma or ductal adenocarcinoma	0	1	1	5 ^c	0	0	0
Total no. of rats with pancreas tumors	5	9 ^a	2	8 ^a	4 ^b	2	1
No. of rats with testicular Leydig tumors	53 ^d	44 ^d	18 ^d	23 ^d	22 ^d	27	74
No. of rats with leukemia or lymphomas	21 ^b	10	2	5	2	6	9
No. of rats with other tumors	24 ^a	22 ^f	10 ^e	15 ^a	20 ^f	10 ^f	51 ^k

^a $P < 0.01$.^b $P < 0.05$.^c Four of these tumors were ductal adenocarcinomas.^d The incidence rates of the testicular Leydig tumors were significantly lower than those in the control (Group VII).^e Prostate *in situ* carcinoma, 9; mammary adenoma, 3; mammary fibroma, 12.^f Prostate *in situ* carcinoma, 11; mammary adenoma, 3; mammary fibroma, 4; mammary adenocarcinoma, 1; thyroid adenocarcinoma, 3.^g Prostate *in situ* carcinoma, 2; mammary adenoma, 2; mammary fibroma, 2; mammary adenocarcinoma, 4.^h Prostate *in situ* carcinoma, 5; adrenal (medullary) tumors, 4; mammary fibroma, 6.ⁱ Prostate *in situ* carcinoma, 6; mammary adenoma, 2; mammary fibroma, 6; mammary carcinoma, 2; thyroid adenocarcinoma, 1; skin papilloma, 2; osteosarcoma, 1.^j Prostate *in situ* carcinoma, 5; mammary adenoma, 1; mammary fibroma, 4.^k Prostate *in situ* carcinoma, 17; mammary adenoma, 15; mammary fibroma, 12; mammary adenocarcinoma, 1; thyroid adenoma, 2; thyroid adenocarcinoma, 4.

Except for a low incidence of pancreatic adenomas, tumor induction in the NG group was not significant compared to controls. In the group treated with NG and NNK, the incidence of lung tumors was significantly greater than in the control group ($P < 0.01$).

The incidence of tumors other than those of the nasal cavity, liver, lung, and pancreas was not higher than controls, indicating that these tumors were not related to *N*-nitrosamine treatment. Compared to controls, the rats in Group I had a higher incidence of leukemia/lymphomas, and the rats in Groups I and II an increase in the total number of mammary tumors.

The number of rats with testicular Leydig tumors was significantly less than controls in all *N*-nitrosamine groups except Group VI.

DISCUSSION

This bioassay resulted in a number of important observations. First, NNK and NNAL, when given in the drinking water, induced large adenomas and adenocarcinomas of the exocrine pancreas. Although exocrine pancreatic tumors have been experimentally induced with synthetic agents (33–37), the present



Fig. 3. Two duct-like pancreas carcinoma nodules in a rat treated with NNK. A rim of normal pancreas surrounds the tumors.



Fig. 5. Duct-like pancreas carcinoma with intraductal papillary projections in a rat treated with NNAL. Normal pancreas at the upper right side.



Fig. 4. Duct-like pancreas carcinoma nodules in a rat treated with NNAL.

results are the first examples of induction of pancreas tumors in laboratory animals with an agent present in tobacco and tobacco smoke. These data are supported by the positive dose-dependent trend in Groups VII, I, and II. The low and insignificant yield of pancreas tumors observed in Group III (5.0 ppm of NNK) may be due to the high incidence of tumors of the lung, nasal cavity, and liver which markedly shortened survival (90.1 ± 11.8 wk) by comparison to the control rats (108.0 ± 11.5 wk). A comparison of the dates of appearance of lung tumors in Group III (5.0 ppm of NNK) and Group II (1.0 ppm of NNK) by the Mann-Whitney *U* test (30) showed a significant delay of onset of tumors in the lower dose group ($P < 0.001$). The highest incidence of benign and malignant pancreas tumors (8 of 30 rats) occurred in the rats receiving 5.0 ppm of NNAL (Group IV), indicating that this enzymatic reduction product of NNK is likely the proximate pancreas carcinogen. Upon i.v. injection in male F344 rats, NNK is rapidly converted to NNAL. The biological half-life of NNK is 0.4 h compared to 2.9 h for NNAL (24). DNA binding studies have shown that NNAL can be reconverted to NNK, yielding the same DNA adducts as those observed upon *in vivo* administration of NNK.⁵ We are studying the role of NNAL in the induction of pancreas tumors by comparing the degree and persistence of DNA adduct formation in the pancreas of rats treated with NNK and NNAL.

In terms of histogenesis, the acinar carcinomas of the pancreas cannot be morphologically traced to the progression towards malignancy of an (acinar) adenoma, although this possibility is not excluded. However, the exocrine nonacinar car-

⁵S. S. Hecht and N. Trushin, *Carcinogenesis* (Lond.) 9: 1665-1668, 1988.



Fig. 6. Low power of a mixed islet and duct pancreas carcinoma nodule. Extensive keratinizing squamous metaplasia. (Rat treated with NNAL.)



Fig. 8. High-power view of the same (Fig. 6) tumor. Tumorous duct with squamous metaplasia in its wall, protruding into the lumen of the duct.



Fig. 7. Enlarged area of squamous keratinizing areas of the tumor of Fig. 6.

cinomas most probably have their origin in the small ducts or ductules, as Pour *et al.* have described for hamsters (33). The cells of the intercalated ductules (Boll ductules), including the centroacinar cells, seem to have regenerative histogenetic capacity similar to the basal (reserve) cells of various mucosal epithelia. The histological images suggest that they can differentiate along various directions forming acinar, ductal, or even Langerhans tissue. Ductular-centroacinar cells seem to be the cells primarily affected by the carcinogen. Regarding the pancreatic tumor containing metaplastic squamous tissue and keratin (Figs. 6 to 8), this is, to our knowledge, the first observation of an experimental carcinoma of the pancreas with epidermoid component. This strengthens the assumption that ductular cells can function as multipotential stem-cells and be the generator of pancreas cancers (36).

One goal of this study was to see whether NG, the major *N*-nitrosamine in betel quid, affects the tumorigenic potency of NNK, which is present in tobacco-containing betel quid and in the saliva of chewers of these quids (16, 22). Betel quid chewing has long been associated with oral cancer in India and many Asian countries. In fact, among the more than 100 listings established in population-based registries around the world, India has the highest rate of oral cancer. So far *N*-nitrosamines are the only known carcinogens in betel quid (4). It was, therefore, important to explore the possible synergistic effect between NG and NNK. When NNK and NG were concomitantly given in the drinking water in a ratio of 1 to 20 ppm (Group VI), tumor yields observed in these rats were not significantly different from those in the rats in Group II (1.0 ppm of NNK). This applied to tumors of the lung, pancreas, nasal cavity, and liver. When NG was applied alone (Group V), the occurrence of acinar tumors of the exocrine pancreas (4 of

30) was significantly higher than in the negative control (Group VII; 1 of 80); however, this isolated finding requires confirmation.

The significant incidence of lung tumors (20 of 80 rats) and the induction of large tumors of the exocrine pancreas (9 of 80) at a total dose of only 15.6 ± 3.3 mg of NNK per rat (Group II) or approximately 0.17 mmol/kg of NNK is important. Even though the incidence rates of tumors of the pancreas and of the lung induced by the lowest dose of NNK (0.073 mmol/kg) were not significant, they were part of significant trends across exposure levels. These results support our hypothesis that NNK is a causative agent for cancers induced in humans by tobacco smoke. In this context one needs to consider the levels of exposure of laboratory animals *versus* those of humans. A smoker of a United States nonfilter cigarette (425 ng of NNK/cigarette; Ref. 38) who smokes 40 cigarettes daily for 40 yr is exposed to about 250 mg of NNK, or approximately 3.6 mg/kg (0.017 mmol/kg). A snuff dipper who consumes 10 g/day of the most popular United States snuff brand (1.8 μ g of NNK/g; Ref. 13) for 40 yr is exposed to about 260 mg of NNK, or approximately 3.7 mg/kg (0.018 mmol/kg). The calculations of human exposure to NNK should be interpreted with caution. They are based on several assumptions. One important consideration is the possibility of endogenous formation of NNK from nicotine upon smoke inhalation or during chewing which likely provides additional carcinogen exposure (9). This study strengthens the need for drastic reduction of the levels of TSNA in all types of tobacco products and in tobacco smoke as long as tobacco usage is highly prevalent throughout the world.

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